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FLUORIMETRY AS A METHOD OF DETERMINING PROTEIN CONTENT OF MILK

SUMMARY

A commercial spectrophotofluorimeter was used to determine the relationship between the intensity of the fluorescence of milk in the ultraviolet region and its protein content. The effect of pH, temperature, milk contaminants, and milk protein aggregation on the fluorescence was studied. By dissociating the milk micelles with a diluent containing citrate, phosphate, and urea, stable fluorescing systems were obtained for study. A double-reciprocal plot of the data obtained showed a good correlation between fluorescence intensity and protein content as determined by Kjeldahl methods. In examining the milk of 88 cows of differing breed, age, and period of lactation it was found that the standard error of the estimate was 0.079% protein. It was concluded that the intensity of fluorescence of milk in the ultraviolet could be used to make accurate and rapid determinations of the protein content of milk, but a number of disadvantages must also be considered before recommending the technique for general use. These include the large dilution of milk samples required, the necessity for rigid temperature control, adverse effects of milk contamination, the relatively small slope of the standard curve, and the fact that commercial fluorimeters suitable for routine use are not available at present.

In 1956, Shore and Pardee in the United States (8) and Vladimirov and Konev (3, 11) in Russia simultaneously published papers demonstrating that part of the radiant energy having a wavelength of 280 mμ was absorbed by protein solutions, then emitted in the form of fluorescence at a wavelength of 340 mμ. Konev continued his investigations of this effect, and in 1960 obtained a Russian patent for a device for determining the protein content of milk by a measurement of the intensity of its ultraviolet fluorescence. His work was brought to the attention of many through a sketchy translation by Lang (5). Recently, Koops and Wijnand (4) verified Konev's basic data, but reported trouble in using equipment of the type described by the Russians. All previous investigators reported scattering in data obtained from a study of the protein content of samples of milk taken from individual cows. Both cited groups reported practically nothing on the various factors governing the intensity of fluorescence from absorbing centers. However, Koops, in one series of determinations, attempted to control the observed drift in fluorescence by the addition of calcium ions to the milk samples.

The method of Konev has also been investigated in the Dairy Products Laboratory of the Eastern Utilization Research and Development Division. This paper concerns itself primarily with a discussion of some factors influencing the fluorescence of absorbing centers in milk, and an estimation of the relative value of fluorimetric measurements for determining the protein concentration of milk.

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Milk samples used in this study were from cows in herds kept at the Agricultural Research Center located at Beltsville, Maryland. During the work, some samples were deliberately selected to represent variations in breed, age, and lactation period.

After mixing, to insure uniform fat distribution, the samples were diluted before analysis. Distilled water and a variety of solutions containing inorganic salts and organic compounds were used as diluents during the course of the investigation. All inorganic materials were of reagent grade; other materials were used as obtained from commercial sources. Since large dilutions had to be made with a high degree of accuracy, a double-dilution technique was employed.

Each of the 88 milk samples from individual cows used in the final appraisal of the method were diluted, using the following procedure:

1) Ten grams of the sample were brought up

to a volume of 100 ml by addition of distilled water.

2) Ten milliliters of this solution were transferred to a 250-ml volumetric flask. Two milliliters of 0.05 M ammonium citrate solution, 20 ml of 0.05 M phosphate buffer, pH 6.8, and 2 ml of a 40% urea solution were added before bringing up to volume with distilled water.

The fluorescence of all diluted samples was measured with an Aminco Bowman [1] Spectrophotofluorimeter (1) equipped with polarizing prisms for scattered light control (10), and sample cells having cross-sectional areas of either 1 cm^2 or 0.0625 cm^2.

The response of the spectrophotofluorimeter was kept constant by adjusting its sensitivity to give the same response to a quinine sulfate solution containing 10 μg/ml in N/10 sulphuric acid. With the instrument used in this study, the meter multiplier was set at 0.1 and the sensitivity adjusted to give a fluorescent response of 58 with the polarizers crossed.

The samples, reagents, and spectrophotofluorimeter were kept in a constant-temperature room. Data were collected at 20 ± 1, unless otherwise specified.

Nitrogen content was determined using a micro-Kjeldahl procedure described in The Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists. All samples were run in duplicate and a study of the results obtained with 180 samples showed the measurements had a precision of one part in 500.

RESULTS

The activation spectrum of fluorescence measured at 340 mμ for milk proteins is shown in Figure 1. This spectrum shows that the principal activation wavelength occurs at about 280 mμ. That peak at 340 mμ is the input radiation scattered at this wavelength.

Figure 2 shows the fluorescent spectrum of milk proteins when the activation wavelength is 280 mμ. The first peak with the polarizers parallel is the scattered radiation of the incident light. The fluorescent radiation reaches a maximum at about 340 mμ. The small peak at about 570 mμ is a second-order harmonic of the scattered radiation at 280 mμ. The effect of crossed polarizers on cancelling out the scattered radiation is obvious.

The effect of pH on the fluorescence of milk proteins is shown in Figure 3. The aggregation leading to precipitation promotes increased fluorescence. Upon short standing, the pH could be reversed across the range studied without hysteresis.

[1] The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U. S. Department of Agriculture.

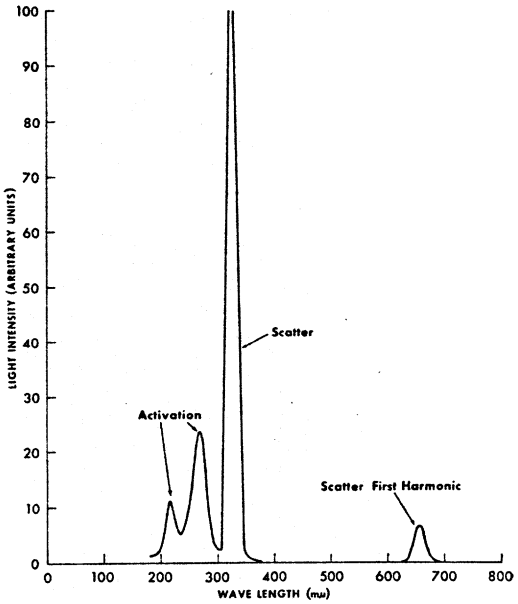

FIG. 1. Activation spectrum of whole milk sample diluted 1:1000 with water and allowed to stand 30 min before scanning. Fluorescence measured at 340 mμ.

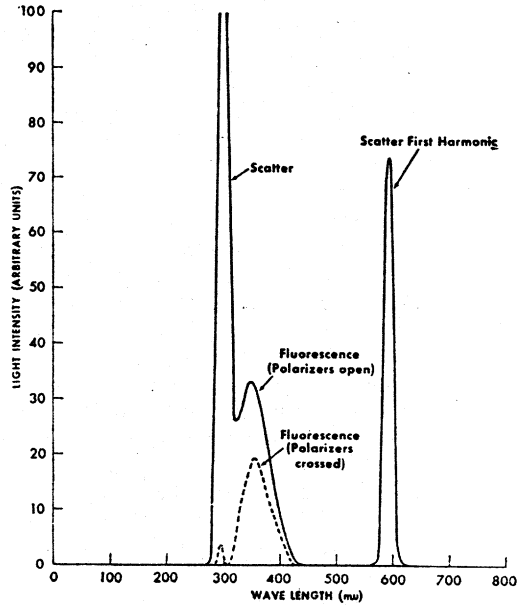

FIG. 2. Fluorescence spectrum of whole milk sample diluted 1:100 with distilled water and allowed to stand 30 min before scanning. Activated with 280 mμ radiation.

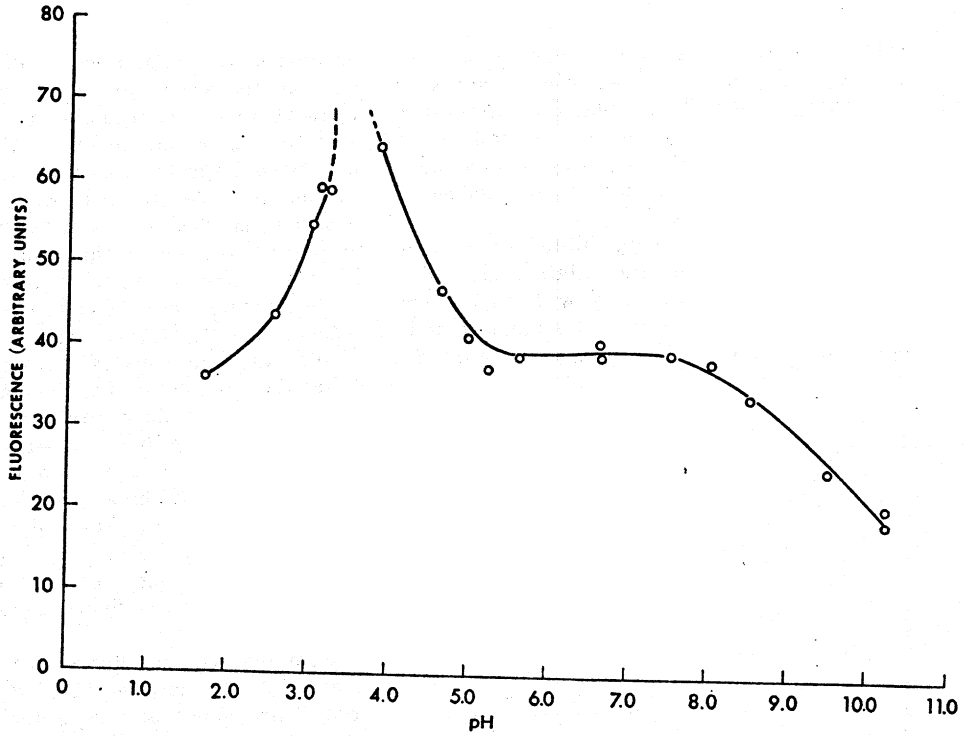

FIG. 3. Effect of pH on fluorescence of whole milk sample diluted 1:250 with distilled water. Activation wave length 280 mμ, fluorescence measured at 340 mμ.

The effect of temperature on fluorescence is shown in Figure 4. A change of 0.7 unit of fluorescence per degree rise in temperature was observed.

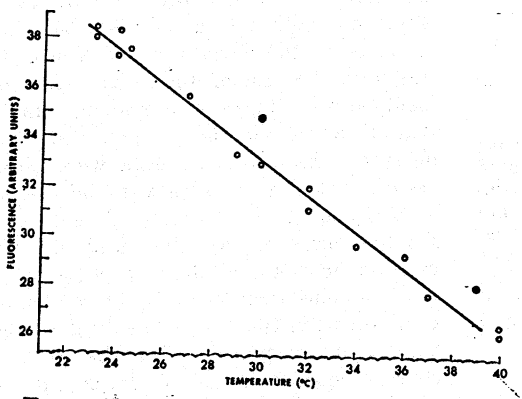

FIG. 4. Effect of sample temperature on fluorescence of whole milk sample diluted 1:250 with distilled water. Activation wave length 280 mμ, fluorescence measured at 340 mμ.

Other factors affecting the fluorescent response are metal ions and organics. Some substances affecting fluorescence and the extent of such effects are given in Table 1. This list is

TABLE 1

Some substances which alter the fluorescence of milk proteins

Compound	Concentration in diluted sample (%)	Fluorescence (arbitrary units)
Control		38.5
$MnSO_4$	0.03	61.0
$CuCl_2$	.2	23.1
$CaCl_2$	0.03	61.7
$Mg(NO_2)_2$	0.03	47.0
$FeSO_4$	0.01	19.9
$Zn(NO_3)_2$	0.02	65.5
$AlK(SO_4)_2 \cdot 12H_2O$	0.04	51.2
Li Acetate	0.01	46.0
Pb Acetate	0.03	59.1
$CeSO_4$	0.02	24.5
KI	Trace	5.0
H_2O_2	1	6.2
Gelatin	0.03	45.6
Gum Arabic	0.03	46.7
Starch	0.04	46.9
Na adipate	0.03	43.5
Tween 60	1	47.0
Ethanol	2	47.1
Tris buffer	1	47.2
P_2O_7	0.04	38.1
Na heptadecyl sulfate		34.6
Hydrazine sulfate		37.8
Nonionic detergent	0.01	66.0

not exhaustive, but it serves to emphasize the need for using clean and well-rinsed glassware in preparing the milk samples for fluorescence measurements. Because of the magnitude of the dilution of the milk proteins, a small amount of a chemical contaminant could produce a large effect in the results.

The fluorescence of milk diluted with water decreased as the time after dilution increased. The decrease in fluorescence with time after dilutions of milk from two individual cows is shown in Figure 5. Here the milks were diluted 1:250 with distilled water. Although the nitrogen content of the two milks was 0.455 and 0.606%, they appeared to lose their fluorescent intensity at similar rates. Examination of this effect at dilutions of 1:500 and 1:1000 showed that the same initial rate prevailed at all dilutions; at the 1:1000 dilution the second part of the curve was much flatter than at the two lower dilutions.

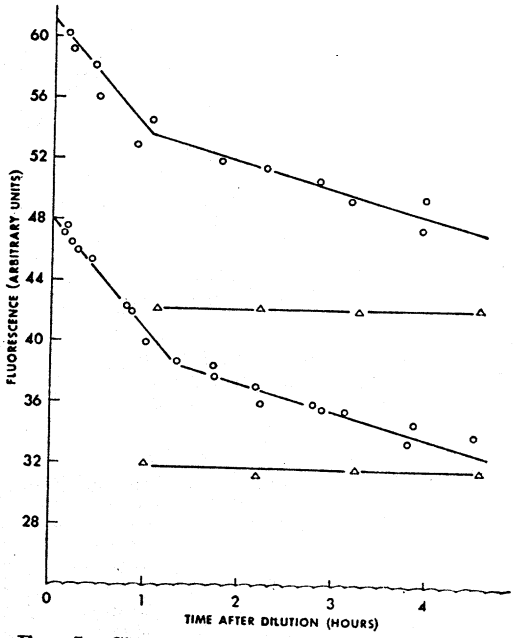

FIG. 5. Change in fluorescence intensity with time lapsed after dilution. All samples diluted 1:250. Open circles represent individual cow samples diluted with distilled water. Triangles are same samples diluted by bringing 10-g milk sample to 100-ml volume, pipetting 10.0 ml of this solution into 250-ml volumetric, adding 2 ml 0.05 M $(NH_4)_2$ H citrate solution, adding 20 ml 0.05 M phosphate buffer solution pH 6.8, and bringing up to volume with distilled water.

The data in Figure 5 represent duplicate results for each milk. From the scatter of the data and its rapid change with time, it was concluded that water as a diluent was not suitable for use in measuring protein concentrations with acceptable precision. Other workers (4) became aware of the effect shown in Figure 5 and tried to prevent its occurrence by adding $CaCl_2$ to their solutions. This approach was tried by us without success. We attempted to stabilize the fluorescence by adding ammonium citrate to sequester the calcium. In less than 10 min after the addition of this reagent, the fluorescence fell to a lower level of intensity and remained constant for at least 4 hr. To eliminate what was apparently adsorption by the dissociated caseinate micelles on the cuvette walls, phosphate buffer at pH 6.8 was added to the milks along with the citrate solution. Without phosphates the postulated adsorption produces a slow upward drift of the observed fluorescence. Figure 5 also shows the stabilizing effect of the citrate-phosphate system on the fluorescence of two milk samples.

With the constant fluorescent response produced by the citrate-phosphate solution, it seemed probable that a reproducible and linear relationship could be obtained between protein concentration and fluorescence. The fluorescence intensity of milks from five individual cows plotted vs. their nitrogen content is shown in Figure 6. Four of the five samples gave a fluorescent response which was linear with concentration on the day the milk was drawn. After the milk was a day old, the fluorescent response of the deviate of the first day was in line with the rest of the milks. This improvement between fluorescent response and concentration when the milks aged was frequently observed. However, no data were obtained to assure that this would always be the case.

Analysis of the possible forces responsible for this observed variation in stability rule out calcium or metal binding, because of the high relative concentration of citrate and phosphate. Consequently, the forces involved in the structural state responsible for the extra fluorescence frequently found in fresh milk must be of the secondary type such as hydrogen or hydrophobic bonds or both. These forces can be dispersed by urea.

Typical results obtained by adding urea to the diluted milk are shown in Figure 7. In all cases, decrease in scattering was observed by use of urea in the diluent system.

When the effect of dilution on the sensitivity of the method was examined using a citrate-phosphate-urea diluent, increased sensitivity was obtained with decreasing dilution as shown in Figure 8. By sensitivity is meant the dif-

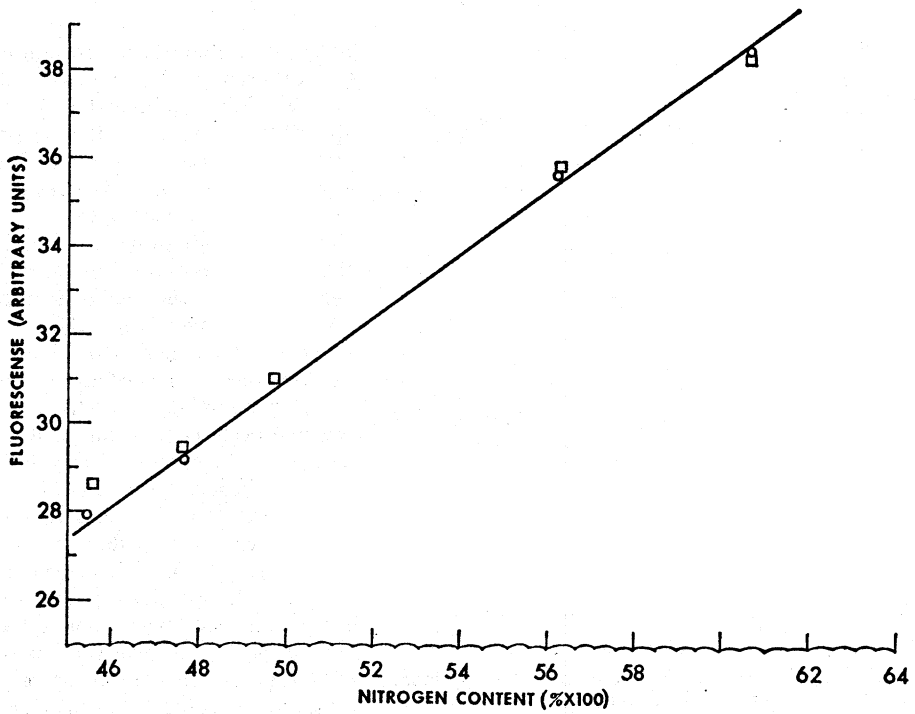

FIG. 6. Effect of aging on samples taken from five individual cows and diluted 1:250 with ammonium citrate–phosphate solution. Open circles represent readings obtained on day of milking; the squares represent values obtained on reanalysis 24 hr later. Activation at 280 mμ, fluorescence measured at 340 mμ.

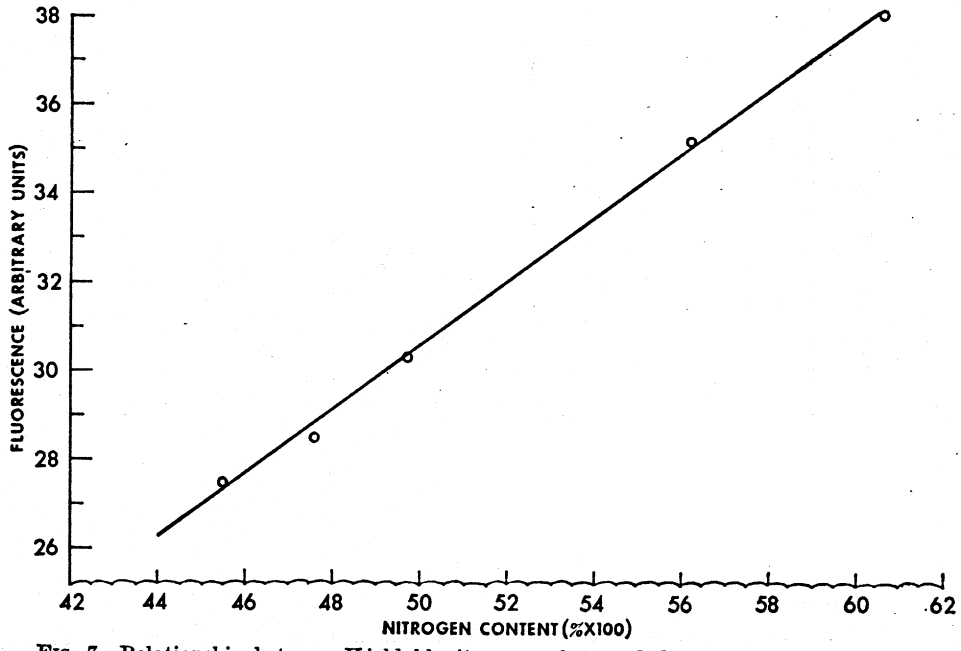

FIG. 7. Relationship between Kjeldahl nitrogen values and fluorescence intensity of milk samples from five individual cows. Samples made up as described in legend of Figure 5, with addition of 2 ml of 40% urea solution before addition of citrate and phosphate. Activation at 280 mμ, fluorescence measured at 340 mμ.

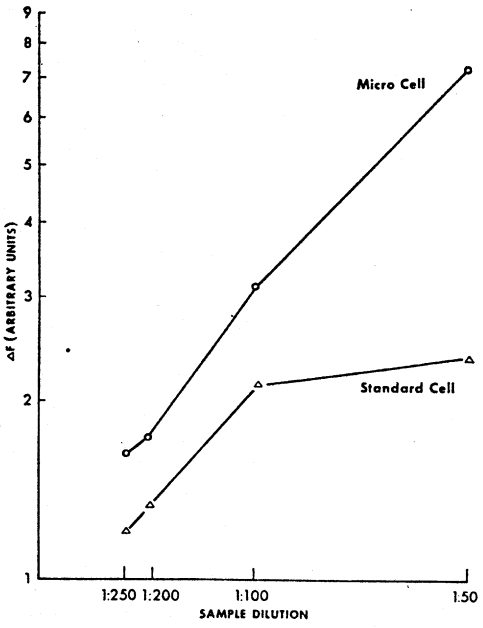

FIG. 8. Effect of dilution and cross-sectional area of cell on the sensitivity ($\triangle$F) of fluorescence measurements made using diluent containing the citrate-phosphate-urea combination described in Figure 7.

ference in fluorescent response, $\triangle$F, between two different milk samples at the different dilutions. With increasing sensitivity a corresponding increase in the scatter of the data was also noted. A sample dilution of 1:250 gave the maximum sensitivity consistent with negligible scatter of data.

The data presented in Figure 8 also indicate that in a standard cell (1 cm^2 cross-section) absorption of the incident light becomes significant. By shortening the solution light path as in the microcell (0.0625 cm^2 cross-section), the number of absorbing centers in it is reduced proportionally. The net effect of using the microcell is that the intensity of the incident light throughout the solution is the same at all dilutions and the fluorescence is then more nearly proportional to the concentration.

When only a few data are considered, as in Figure 7, they are described satisfactorily by a direct plot of fluorescence vs. nitrogen concentration. However, when a considerable number of data are involved, as in Figure 9, a curvature of the fluorescent response with concentration is observed. Such a curvature suggests that a double-reciprocal plot of the data would be more suitable. Such a plot of the data

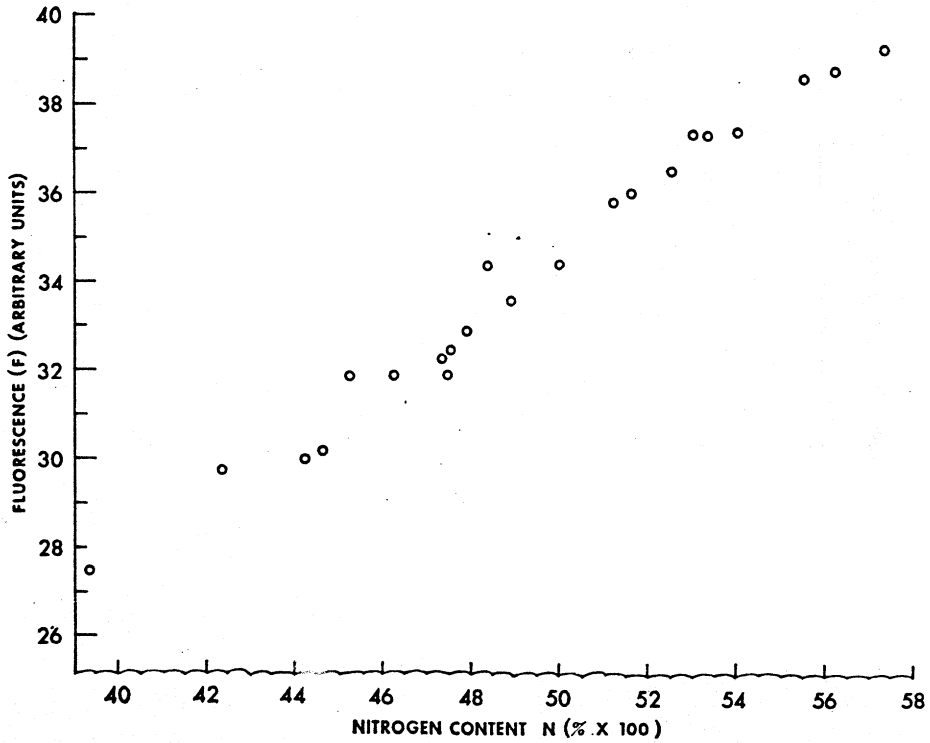

FIG. 9. Relationship between Kjeldahl nitrogen values and fluorescence intensity of milk samples taken from individual Holstein cows. All samples diluted with citrate-phosphate-urea combination as described in legend of Figure 7.

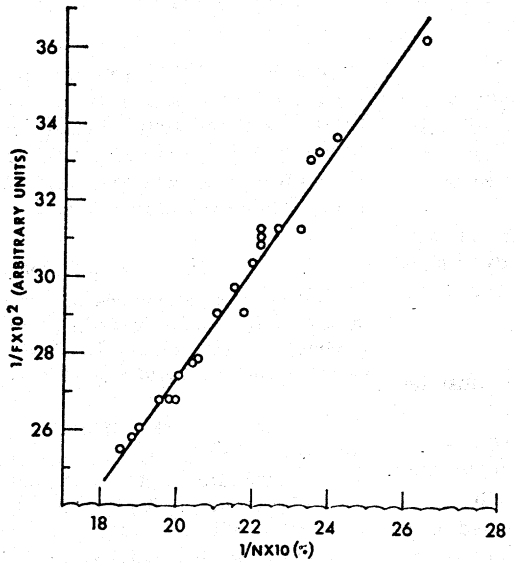

FIG. 10. A double-reciprocal plot of data in Figure 9.

is shown in Figure 10. The value of this type of plot was further demonstrated in a study of the milk of 88 individual cows representing Holstein, Jersey, Guernsey, Ayrshire, and Brown Swiss breeds and of cows which were crosses of Holstein, Ayrshire, and Brown Swiss, all of varying age and stage of lactation. The standard error of the estimate for this lot was 0.079% protein—when compared with protein concentration calculated from micro-Kjeldahl nitrogen determinations. When these data were plotted with fluorescence as a function of concentration, the standard error would have been perhaps as great as 0.15%. In this study of 88 cows no correlation was found between the fluorescence/unit protein and the breed, period of lactation, or age of the cow. It was also found that a high order of precision could be obtained by observing fluorescence through the crossed polarizers only. All other fluorescence data presented in this paper represent the sum of galvanometer readings observed with the polarizers in the crossed and in the open position. This summing was found to reduce observed scattering of data slightly, but is bothersome when analyzing large numbers of samples.

DISCUSSION

The intensity of the ultraviolet fluorescence of milk exposed to 280 mμ wavelength radiation should be proportional to the tyrosine and tryptophan content of the proteins in milk. Even though the observed fluorescence spectrum is characteristic of pure tryptophan, the contribution of the tyrosine cannot be ruled out (9).

The efficiency with which these absorbing centers convert absorbed energy into ultraviolet radiation is influenced by a variety of factors.

Our study shows that factors influencing the fluorescence of milk proteins can be divided into two groups:

1. Those factors which affect all fluorescing substances in general, such as temperature, pH, salt concentration, and quenching agents.
2. Certain factors which are unique to milk proteins and appear largely to be a consequence of their structural character.

The unique protein structure in milk which influences fluorescence is the casein micelle. Higher levels of fluorescence would be observed if this structure could be stabilized against change on dilution. We were unable to do this satisfactorily and found that the best determination of protein concentration by fluorescence measurements could be made after dissociating the micelles with a diluent containing agents capable of sequestering calcium and rupturing hydrogen and hydrophobic bonds. The use of low levels of urea in dilute aqueous systems suggests that the casein micelle structure maintains its integrity through hydrophobic as well as ionic bonds.

The data obtained from observations of systems devoid of urea suggest that individual cows may produce milk in which the secondary forces involved in casein bonding may vary considerably.

Aside from the effect of structural features of the casein micelle, another effect requiring consideration is the relative proportion of the various proteins in milk samples. The magnitude of the change in fluorescence to be expected from this last source can be calculated from the reported amino acid content of the different milk proteins, as determined by Gordon and co-workers and Brand (2) and from the differences in concentration observed in the different proteins due to breed and period of lactation (6, 7). Such calculations show a possible difference between protein content calculated from per cent nitrogen and fluorescence of only 0.1% protein ± 0.05%. These calculations appear to be corroborated by the experimental data reported here. Or, the calculated error from this source is less than the experimental error of the method described in this paper.

CONCLUSIONS

From the data obtained in this study it was concluded that the protein content of milk samples could be determined with a high degree of accuracy by use of fluorescence measurements.

In considering the applicability of the method to routine analysis, the following advantages and disadvantages of the technique must be considered:

A. Disadvantages

1. Large dilutions of milk samples must be accurately made.
2. Precautions must be taken to avoid sample contamination. Even detergents can affect fluorescence of milk proteins.
3. The standard curve for the method has a relatively small slope. Small errors in fluorescence measurements are reflected in large protein-concentration errors.
4. Temperature of samples must be rigidly controlled during analysis.
5. Commercial fluorimeters suitable for routine operations are not available at present.

B. Advantages

1. Turbid solutions can be analyzed directly. It is not necessary to filter or centrifuge samples before analysis.
2. The property directly measured is that of the native protein molecules, not that of an intermediate material of dubious composition or purity.
3. If proper equipment is designed, direct reading of the protein concentration of the milk on a single calibrated meter would make the method a rapid one.

It seems probable that variation in instrumental response is the factor most responsible for remaining observed deviations in the fluorescence data. Fluorescence varies directly as the intensity of the light source. With the particular instrument used there was no way of detecting a change in the intensity of the light source immediately before making a measurement. A change in the intensity sufficient to cause a deviation of one unit of fluorescence as measured with crossed polarizers corresponds to ca. 0.19% protein on a double-reciprocal plot. Consequently, a constant-light intensity is mandatory. When the incident-light intensity remained constant, the fluorescence with crossed polarizers could be read with a precision of less than 0.2 unit.

ACKNOWLEDGMENT

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